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Published in:
The Journal of Biological Chemistry

DOI:
[10.1074/jbc.M500522200](https://doi.org/10.1074/jbc.M500522200)

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Document Version
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Publication date:
2005

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Citation for published version (APA):

Schuurman-Wolters, G. K., & Poolman, B. (2005). Substrate Specificity and Ionic Regulation of GlnPQ from *Lactococcus lactis*. An ATP-Binding Cassette Transporter with Four Extracytoplasmic Substrate-Binding Domains. *The Journal of Biological Chemistry*, 280(25), 23785 - 23790.
<https://doi.org/10.1074/jbc.M500522200>

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Substrate Specificity and Ionic Regulation of GlnPQ from *Lactococcus lactis*

AN ATP-BINDING CASSETTE TRANSPORTER WITH FOUR EXTRACYTOPLASMIC SUBSTRATE-BINDING DOMAINS*

Received for publication, January 14, 2005, and in revised form, March 23, 2005
Published, JBC Papers in Press, March 28, 2005, DOI 10.1074/jbc.M500522200

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We report on the functional characterization of GlnPQ, an ATP-binding cassette transporter with four extracytoplasmic substrate-binding domains. The first predicted transmembrane helix of GlnP was cleaved off in the mature protein and most likely serves as the signal sequence for the extracytoplasmic substrate-binding domains. Deletion analysis showed that the substrate-binding domain, in the primary sequence of GlnP nearest to the translocator domain, is used as the receptor that delivers the substrate to the translocator. Membrane reconstitution of the detergent-solubilized and purified GlnPQ complex yielded proteoliposomes that transported glutamine and glutamic acid at the expense of ATP. The transport activity of GlnPQ increased with luminal salt concentration and internal pH, but the mechanism of ionic activation of the transporter is distinct from that of other osmoregulatory ATP-binding cassette transporters and does not depend on the presence of anionic lipids. The regulation of GlnPQ conforms to an electrostatic switch in which protein domain(s) and low molecular weight electrolytes participate.

The basic unit of ATP-binding cassette (ABC)¹ transporters consists of two translocator domains and two ATP-binding cassettes, together referred to as the translocator (1). The domains of the translocator can be present as separate subunits or fused to each other in any possible combination. In addition, prokaryotic ABC transport systems involved in solute uptake employ an accessory protein that captures the ligand and delivers it to the translocator. These so-called substrate-binding proteins (SBPs) are present in the periplasm of Gram-negative bacteria, or they can be tethered to the membrane via a lipid or protein anchor (Gram-positive bacteria, Archaea). Recently, we discovered that members within two families of the ABC superfamily,

which are the OTCN and PAO families, can have the SBPs fused to the translocator domains (2). Within the OTCN family, one or two domains fused in tandem are present at the carboxyl terminus of the translocator subunit. Within the PAO family, one or two domains are linked to the amino-terminal end of the translocator subunit, and these are preceded by a predicted signal sequence. Two of these chimeric substrate-binding/translocator proteins along with two ATP-binding cassettes form the functional unit for transport, and these systems thus have two or four substrate-binding domains.

Previously, we have described the properties of the osmoregulatory glycine betaine transporter (OpuA) from *Lactococcus lactis* (3, 4), which has two substrate-binding domains in the functional complex. We now report the cloning and amplified expression of the *glnPQ* genes, encoding an ABC transporter with four substrate-binding domains. The domain structure and membrane topology of the GlnPQ system is schematically depicted in Fig. 1A. The GlnPQ complex has been purified and reconstituted in proteoliposomes, allowing us to determine the rather unique substrate specificity and salt and pH dependence of the system. We show that GlnPQ corresponds to the previously characterized glutamine-glutamate transport system from *L. lactis*, which has the unusual feature of transporting glutamic acid rather than glutamate anion, the dominant species at physiological pH values (5, 6). We also show that the first 26 amino acids of the GlnP open reading frame are cleaved off in the mature protein and, apparently, serve as signal sequence for the translocation of the two amino-terminal substrate-binding domains.

EXPERIMENTAL PROCEDURES

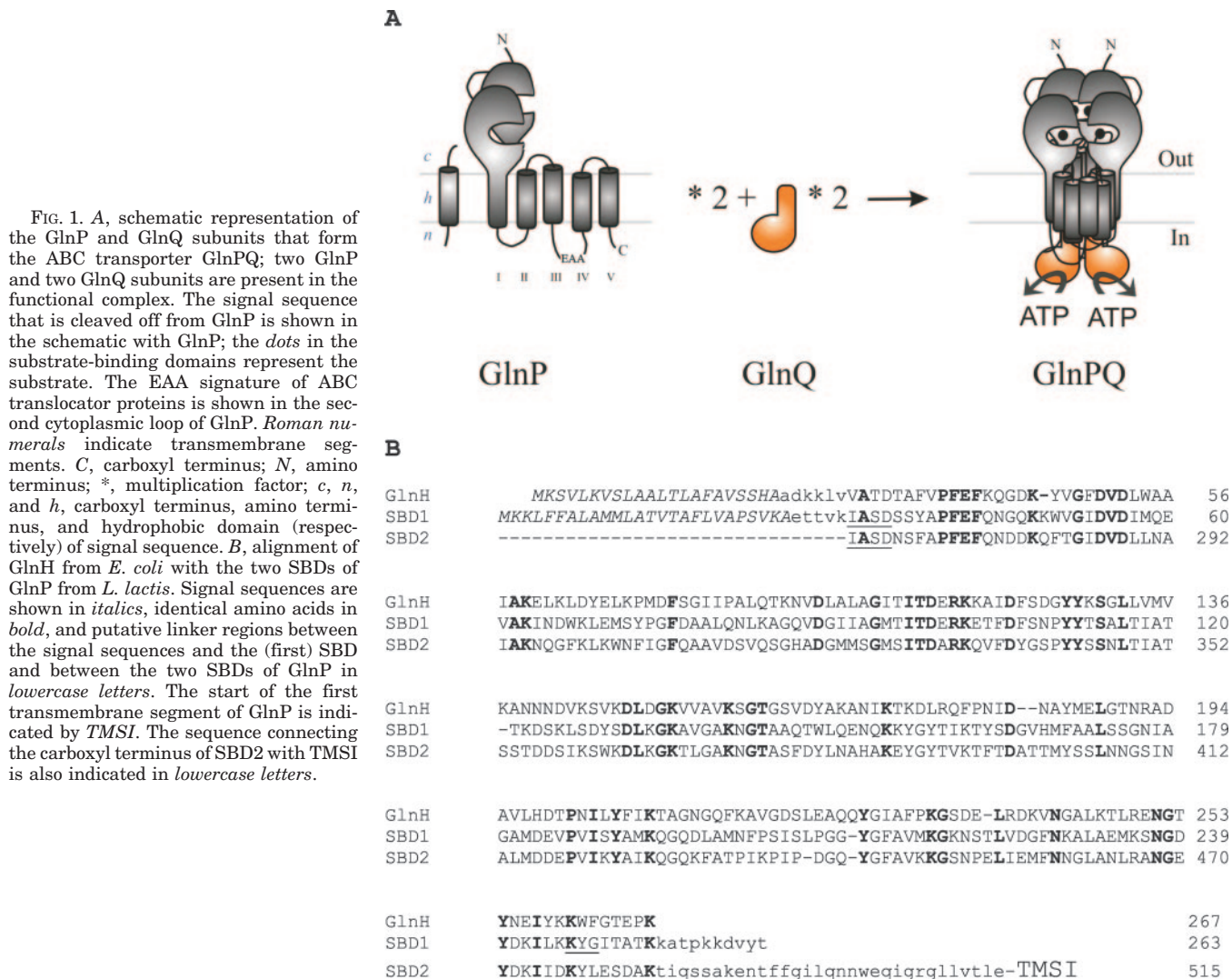
Materials—M17 broth was obtained from Difco, MRS broth from Merck, nickel-nitrilotriacetic acid resin from Qiagen Inc., *N*-dodecyl- β -D-maltoside from Anatrace Inc., Triton X-100 from Roche Applied Science, Biobeads SM-2 from Bio-Rad, total *Escherichia coli* lipid extracts, *L*- α -phosphatidylcholine from egg yolk, and synthetic lipids (dioleoyl-phosphatidyl derivatives: DOPE, DOPG, and DOPC) from Avanti Polar lipids. Radiolabeled L-[¹⁴C]glutamine (256 mCi/mmol), L-[¹⁴C]glutamate (256 mCi/mmol), and L-[¹⁴C]arginine (310 mCi/mmol) were obtained from Amersham Biosciences. Creatine phosphate was obtained from Sigma. Creatine kinase and ATP were obtained from Roche Applied Science. All other chemicals were of analytical grade and obtained from commercial strains.

Bacterial Strains, Growth Conditions, and Membrane Vesicle Preparation—*L. lactis* strain NZ9000 was cultivated semi-anaerobically at 30 °C in a medium containing 5% (w/v) whey permeate, 0.5% (w/v) yeast extract plus 65 mM sodium-phosphate, pH 7, supplemented with 0.5% (w/v) glucose and 5 μ g/ml chloramphenicol when carrying pNZglnPQhis or other derivatives. For the preparation of membrane vesicles, cells were grown in a 15-liter pH-regulated fermentor at pH 6.5 to an A_{600} of 1–2, after which the transcription from the *nisA* promoter was switched on by the addition of 0.01% (v/v) culture supernatant of the Nisin A-producing strain NZ9700. The cells were harvested, and the mem-

* This work was supported by a “Top-subsidie” grant (NWO-CW, Grant 700-50-302). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: ABC, ATP-binding cassette; SBP, substrate-binding protein; SBD, substrate-binding domain; DDM, dodecyl- β -D-maltoside; K-MES, potassium-2-(*N*-morpholino)ethanesulfonic acid; DOPE, dioleoyl-phosphatidylethanolamine; DOPG, dioleoyl-phosphatidylglycerol; DOPC, dioleoyl-phosphatidylcholine.



brane vesicles were prepared according to standard procedures (3). When appropriate, a chemically defined medium was used to cultivate the cells, as described previously (18).

Disruption of Chromosomal *glnP*—The *glnP* gene of *L. lactis* JIM7049, a derivative of IL1403, was disrupted by plasmid integration via homologous recombination using an 886-bp EcoRI fragment of the *glnP* gene (bp 824–1710) ligated in the integration vector pOri28 (erythromycin-resistant), yielding pOri28disP. *L. lactis* LL108, carrying the *repA* gene in *trans*, was used as the host for the construction and propagation of the vector. Subsequently, *L. lactis* JIM7049 was transformed with pOri28disP to inactivate the *glnP* gene. Transformants were selected for growth on GM17 agar supplemented with 2.5 µg/ml erythromycin. The integration of the vector was verified by PCR, and the disruption strain was designated JIM7049disglnP.

Plasmid Construction—For the overexpression of the GlnPQ complex from *L. lactis* IL1403 (GenBank™ accession numbers NP_267915 for *glnP* and NP_267916 for *glnQ*), the genes were placed under the control of the *nisA* promoter (7). The genes were amplified by PCR, using as the forward primer (*glnPQfor*) 5'-GAGGAGCCATGGGAAGAAAT-TATTTTCGCTCTG-3', starting at the ATG of *glnP*, and as the reverse primer (*glnPQrev*) 5'-AATGGGTCTAGACCGATCCTGCATTCAAA-ACTTTTGATAA-3', ending at the stop of *glnQ*. The NcoI site in the forward primer and the BamHI site in the reverse primer are underlined. The product was ligated into the NcoI/BamHI site of pNZOpuAAhis, thereby replacing the *opuAA* gene (3). The result of this strategy is the insertion of a glycine after the amino-terminal methionine of the GlnP protein and the addition of a His₆ tag at the carboxyl terminus of the GlnQ protein.

For the construction of the deletion mutants ΔSBP1 and ΔSBP2, we took advantage of the homology between the two domains and the identity with GlnH from *E. coli*, for which crystal structures are available (8, 9; see also Fig. 1B). For ΔSBP1 (deletion of amino acids 33–265),

two silent NruI sites were introduced into the *glnP* gene, one in the amino acid sequence 32–34(IAS) using *glnPQfor* as the forward primer and 5'-CGTATGAGCTATCGCTCGCGATTTTACAGTTGTCTCG-3' as the reverse primer, and one at residues 264–266(IAS) using 5'-CC-TAAAAAAGATGTTTACACCATCGCGAGCGATAATAGCTTC-3' as the forward primer and *glnPQrev* as the reverse primer. The two PCR products were subjected to restriction with NruI, followed by ligation. After purification, the ligation product was amplified with *glnPQfor* and *glnPQrev* and ligated into the NcoI and BamHI restriction sites of pNZglnPQhis, yielding pNZglnPQhisΔSBP1. The strategy for preparing ΔSBP2 was essentially the same, except that ScaI sites were introduced 5' and 3' of the region specifying the second substrate-binding domain. The first ScaI site was made by changing the sequence (residues 246–248) KYG into KYL, using *glnPQfor* as forward and 5'-GCTTTTGTAGTGGCAGTAATTAAGTACTTTTGTAGGATTTTATCA-TAATCTCCG-3' as the reverse primer. For the second ScaI site, the sequence corresponding to amino acid 478 and 480 (KYL) was modified using 5'-GGTGAATATGATAAAATCATTGATAAGTACTTTAGAGAGT-GATGC-3' as the forward and *glnPQrev* as the reverse primer. The resulting plasmid was named pNZglnPQhisΔSBP2; ΔSBP2 corresponds to a deletion of residues 248–480.

Whole Cell Uptake Experiments—*L. lactis* strain JIM7049 was grown on MRS medium plus 2% w/w glucose to an A_{600} of 0.6–1 and harvested by centrifugation (10 min, 4,000 × g). The cells were washed three times with ice-cold 50 mM potassium phosphate, pH 6.5, and suspended in this buffer to an A_{600} of 100. For the transport experiments, the cells were diluted to an A_{600} of 5 into 50 mM potassium phosphate, pH 6.5, 5 mM MgCl₂ plus 10 mM glucose and allowed to equilibrate at 30 °C. After 3 min of incubation, the uptake was started by adding substrate ([¹⁴C]glutamine, [¹⁴C]glutamate or substrate + inhibitor). The final protein concentration in the assays was 1 mg/ml. At given time intervals, 40-µl samples were taken and

diluted with 2 ml of ice-cold 50 mM potassium phosphate, pH 6.5. The samples were collected on 0.45- μ m pore size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassel, Germany) and washed twice with 2 ml of the same buffer. The radioactivity on the filters was determined by liquid scintillation spectrometry.

Purification of GlnPQ—Membrane vesicles were resuspended in 50 mM potassium phosphate, pH 8.0, 200 mM KCl, 20% glycerol (buffer A) to a concentration of 5 mg of protein/ml and solubilized with 0.5% DDM for 30 min on ice. After centrifugation, the supernatant was incubated with nickel-nitrilotriacetic acid resin (0.5 ml of resin/10 mg of membrane protein) for 1 h, rotating at 4 °C in the presence of 10 mM imidazole. Subsequently, the resin was washed with 20 column volumes of buffer A supplemented with 0.05% DDM and 10 mM imidazole. The His-tagged proteins were eluted from the column with 3 column volumes of buffer A supplemented with 0.05% DDM and 200 mM imidazole.

Membrane Reconstitution of GlnPQ—Liposomes composed of egg yolk L- α -phosphatidylcholine and purified *E. coli* lipids in a 1:3 (w/w) ratio were prepared, and membrane reconstitution of the GlnPQ complex was performed essentially as described by Knol *et al.* (10), except that 20% (v/v) glycerol was added to the buffers. Briefly, preformed liposomes (4 mg/ml) were destabilized by titration with Triton X-100, and the turbidity of the suspension at 540 nm was used to monitor the physical state of the liposomes. Detergent-saturated liposomes were mixed with purified GlnPQ complex in a 100:1 ratio (w/w) and incubated for 30 min at room temperature under gentle agitation. To keep the molar lipid-to-protein ratio the same as for the wild-type protein complex, purified GlnPQ Δ SBP1 and GlnPQ Δ SBP2 were reconstituted at 128:1 (w/w) lipid:protein. To remove the detergent, Biobeads SM-2 were added at a wet weight of 40 mg/ml, and the sample was incubated for another 15 min. Fresh Biobeads SM-2 (40 mg/ml) were added to the sample four times, and the incubations were continued at 4 °C for 15 min, 30 min, overnight, and 1 h, respectively. After 5 \times dilution into 50 mM potassium phosphate, pH 7.0, the proteoliposomes were collected by centrifugation (150,000 \times g for 90 min) and stored in liquid nitrogen. For membrane reconstitution in defined lipid mixtures, synthetic lipids (DOPC, DOPG, and DOPE) dissolved in chloroform were mixed in the appropriate ratio, and after evaporation of the solvent, the lipids were washed with ethanol and dissolved in 50 mM potassium phosphate, pH 7.0. After sonication with a probe sonicator (15 s on/45 s off, 6 cycles at an intensity of 4 μ m), the liposomes were frozen/thawed three times and stored in liquid nitrogen. The protocol used for incorporation of GlnPQ into these liposomes was essentially the same as described for the *E. coli* lipid/egg phosphatidylcholine mixture.

ATP-driven Uptake in Proteoliposomes—An ATP regenerating system, consisting of creatine kinase (2.4 mg/ml), ATP (9 mM), MgSO₄ (9 mM), and creatine phosphate (24 mM), together with 50 mM potassium phosphate, pH 7.0, was enclosed in the proteoliposomes by two freeze/thaw cycles. Following extrusion of the proteoliposomes through a polycarbonate filter (400-nm pore size), the proteoliposomes were collected by centrifugation (300,000 \times g for 15 min) and washed twice and resuspended in 90 mM potassium phosphate, pH 7.0, to a concentration of 20 mg of lipid/ml. Uptake was measured in 90 mM potassium phosphate, pH 7.0, either or not supplemented with 250 mM KCl as specified in the figure legends. This medium, supplemented with the appropriate substrate, was incubated at 30 °C for 2 min prior to adding proteoliposomes kept on ice to a final concentration of 1 mg of lipid/ml. At given time intervals, 40- μ l samples were taken and diluted with 2 ml of ice-cold isotonic buffer (90 mM potassium phosphate, pH 7.0, either or not supplemented with 250 mM KCl). The samples were collected on 0.45- μ m pore size cellulose nitrate filters and washed twice as described above.

Manipulation and Determination of the Internal pH—Proteoliposomes were equilibrated with 10 mM potassium phosphate, 100 mM potassium acetate, pH 6.5, the ATP-regenerating system, and 0.5 mM of the pH indicator probe pyranine. After two freeze/thaw steps and extrusion through a 400-nm filter, the proteoliposomes were collected by centrifugation (300,000 \times g for 15 min), washed four times in 50 mM potassium phosphate, 100 mM potassium acetate, pH 6.5, to remove external pyranine, and suspended to 40 mg/ml lipid. The internal pH was varied by diluting the proteoliposomes in isotonic medium with varying ratios of potassium acetate and potassium-2-(*N*-morpholino)ethanesulfonic acid (K-MES). The internal pH was followed by measuring the fluorescence of pyranine (excitation at 465 \pm 2 nm and emission at 511 \pm 5 nm) in a volume of 1 ml. At the end of each experiment, 0.5 μ M nigericin was added to dissipate any pH gradient and the fluorescence signal was correlated to the pH by measuring the pH upon the addition of small amounts of KOH or HCl.

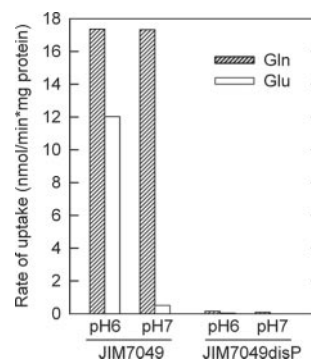


FIG. 2. Uptake of glutamine and glutamate in *L. lactis* JIM7049 (carrying pGKhis) and JIM7049disP. Initial rates of uptake at 20 μ M substrate were determined for glutamine and glutamate at pH 6 or 7.

Amino Acid Sequencing of GlnP—To determine the amino-terminal amino acid sequence of GlnP, the purified complex was run on an SDS-PAGE gel, and the appropriate band was sliced out. Amino-terminal sequencing was carried out by Eurosequence B.V., Groningen, The Netherlands.

Miscellaneous—The protein concentration of membrane vesicles was determined by the method of Lowry *et al.* (19), using bovine serum albumin as a standard. The concentration of the purified GlnPQ complex was determined using calculated molar absorption coefficients of 83,200 mol⁻¹·cm⁻¹ for GlnP and 1,615 mol⁻¹·cm⁻¹ for GlnQ, respectively.

RESULTS

Gene Disruption—Integration of *pOri28disP* into the chromosome of *L. lactis* JIM7049 led to disruption of the *glnP* gene and abolished the uptake of glutamine and glutamate (Fig. 2). Although glutamine (or glutamate) is essential for growth of *L. lactis*, the disruption mutant was able to grow in complex MRS broth, probably because of the presence of glutamine- and/or glutamate-containing peptides. Using a chemically defined medium (18) supplemented with glutamine or glutamate as the sole source for glutamine or glutamate, growth was not observed for the disruption mutant, but growth in chemically defined medium could be rescued by the addition of the dipeptide alanine-glutamine, which is taken up by a peptide transport system (11). These experiments strongly indicate that GlnPQ is the sole transport system for the uptake of glutamine and glutamate, which is consistent with previous observations that these amino acids compete for transport in *L. lactis*. In these experiments, it was also shown that glutamine and glutamic acid (and not the glutamate anion) are the actual substrates of, at that time, unknown transporter(s) (6).

Amplification and Purification of GlnPQ—The genes encoding the translocator subunit (GlnP) and the ATPase (GlnQ) were placed in tandem in the pNZOpuAA plasmid, using the NcoI and BamHI restriction sites. Hereby, an extra glycine residue is introduced into GlnP as the second amino acid, and a His₆ tag is added to the carboxyl terminus of GlnQ. After nickel-nitrilotriacetic acid purification, the GlnPQ complex was separated on a SDS-polyacrylamide gel (Fig. 3), and the GlnP band was sliced out for amino-terminal amino acid sequencing. The amino-terminal sequence started with ETTVKI, demonstrating that GlnP is synthesized as pre-protein with an amino-terminal signal sequence corresponding to MKKLF-FALAMMLATVTAFLVAPSVKA (Fig. 1B).

Substrate Specificity of GlnPQ and the Role of the Individual Substrate-binding Domains—Glutamine uptake followed Michaelis-Menten kinetics (Fig. 4A), from which an apparent *K_m* for uptake of 6.3 \pm 0.8 μ M was estimated. Using 5 μ M [¹⁴C]glutamine as the reporter ligand, we tested all natural L-amino acids (except Tyr), the dipeptide Ala-Gln and methyl-

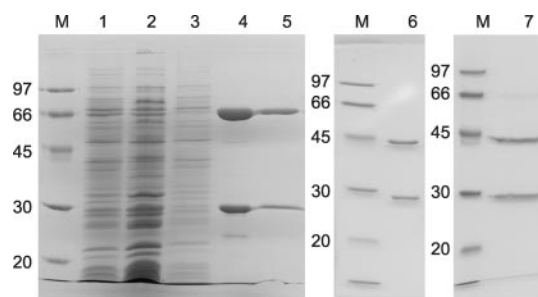


FIG. 3. Purification of GlnPQ complex from membrane vesicles. Lanes M, markers; lane 1, solubilized membranes (23 μ g of total protein); lane 2, supernatant after ultracentrifugation; lane 3, flow-through from nickel-nitrilotriacetic acid column; lane 4, peak fraction of GlnPQ after elution from nickel-agarose (3.4 μ g protein); and lane 5, membrane-reconstituted GlnPQ complex in *E. coli* phospholipid/egg phosphatidylcholine liposomes (1.4 μ g protein). Lanes 6 and 7, purified GlnPQ Δ SBD1 and GlnPQ Δ SBD2 (1 μ g of protein). Numbers to the left of each panel are measurements in kDa.

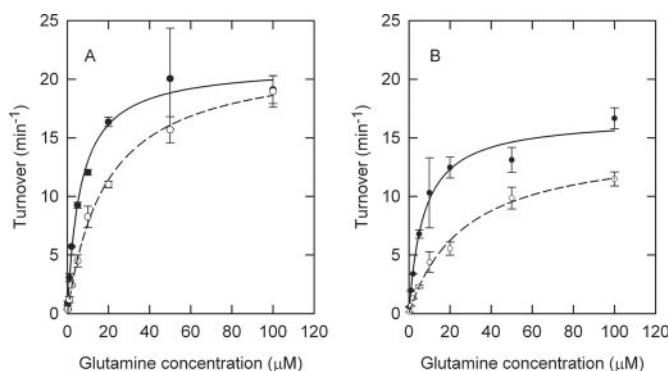


FIG. 4. Kinetics of [14 C]glutamine uptake in proteoliposomes by wild-type GlnPQ (A) and GlnPQ Δ SBD1 (B). Initial rates of transport were determined from the linear increase in uptake over the first 70 s, and data were fitted using the Michaelis-Menten equation. Uptake was performed without (closed circles, solid line) and in the presence (open circles, dotted line) of 5 mM L-arginine. The assay buffer was 90 mM potassium phosphate + 250 mM KCl, pH 7.0, and experiments were started by adding proteoliposomes from ice; [14 C]glutamine concentrations between 200 nM and 100 μ M were used.

ammonium as possible substrates at 1 and 10 mM concentration. Significant inhibition of glutamine transport was only observed in the presence of glutamate, arginine, and γ -glutamylhydrazide (data not shown). The inhibition by arginine and γ -glutamylhydrazide was competitive, with K_i values of 2.2 ± 1.1 mM ($n = 4$; Fig. 4A) and 80 ± 50 μ M ($n = 3$), respectively, where n is the number of independent measurements. Competitive inhibition of glutamine transport by glutamate and uptake of [14 C]glutamic acid was only observed at low pH (pH < 6), which most likely reflects the low fraction of glutamic acid at the ambient pH values; the pK_a of the γ -carboxyl group is 4.25. Uptake of arginine could not be measured, possibly due to the low affinity of the transporter for arginine, but it could not be ruled out that arginine is only inhibiting and not transported. In summary, these measurements, together with the pH dependence of transport (see below), reveal the unusual substrate specificity of the transporter, which is that GlnPQ transports glutamine and glutamic acid and is competitively inhibited by arginine and γ -glutamylhydrazide.

By aligning the sequences of the two substrate-binding domains (SBDs) of GlnPQ with the corresponding substrate-binding protein GlnH from *E. coli* and inspection of the crystal structures of GlnH (8, 9), we deduced a linker of ten residues that connects SBD1 with SBD2 (Fig. 1B, katpkkdvyt). To construct the transporter complex lacking the first substrate-binding domain (GlnPQ Δ SBD1), the sequence ettvk following the

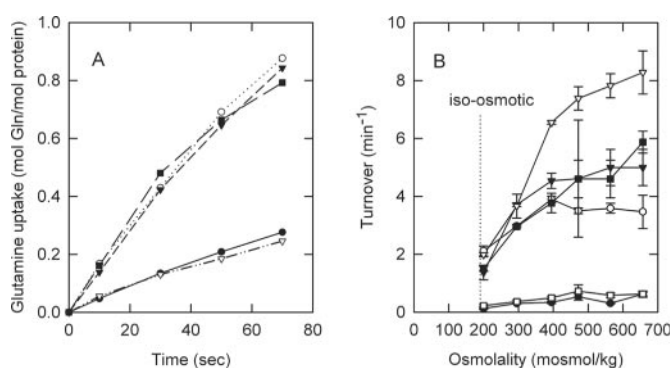


FIG. 5. Effect of osmotic activation and membrane lipid composition on membrane-reconstituted GlnPQ. A, uptake of [14 C]glutamine (final concentration of 1 μ M) was performed in 90 mM potassium phosphate, pH 7.0 (basal medium), under iso-osmotic (closed circles) and hyperosmotic conditions (500 mosmol/kg) by adding 250 mM KCl (open circles), 250 mM NaCl (closed squares), 363 mM sucrose (closed triangles) or 363 mM glycerol (open triangles) to the basal medium. B, uptake of [14 C]glutamine (final concentration of 5 μ M) was assayed in 90 mM potassium phosphate, pH 7.0, plus varying amounts of KCl. The liposomes used for reconstitution consisted of 50 mol % DOPE and 0 (closed circles), 5 (open circles), 17 (closed triangles), 34 (open triangles), 45 (closed squares), and 50 mol % DOPG (open squares) plus 50, 45, 33, 16, 45, and 0 mol % DOPC, respectively.

signal sequence was fused to the first conserved residue (Ile) in SBD2, resulting in a mature GlnP Δ SBD1 protein that started with the sequence ETTVKIASDNSF (which compares to ETTVKIASDSSY) in wild-type GlnP. For the construction of GlnPQ Δ SBD2, the sequence YDKILKKY (just before the end of SBD1) was fused to the sequence starting with LESDAKT, leaving the last seven amino acids of SBD2 in place. The expression, purification, and membrane reconstitution of GlnPQ Δ SBD1 and GlnPQ Δ SBD2 was comparable with wild-type GlnPQ (Fig. 3). However, with GlnPQ Δ SBD2, uptake of glutamine or glutamate was no longer observed (data not shown). On the contrary, the kinetics of glutamine uptake ($K_m = 7.2 \pm 1.2$ μ M) and arginine inhibition ($K_i = 1.8$ mM) of GlnPQ Δ SBD1 was similar to that of the wild-type complex (Fig. 4B), indicating that the SBD, in sequence nearest to the translocator domain, is used to deliver the substrate to the translocator. The turnover number of GlnPQ Δ SBD1 was 20–30% lower than that of wild-type GlnPQ, as deduced from activity measurements in several independent preparations of proteoliposomes (Fig. 4 and data not shown).

Salt and Lipid Dependence—After reconstitution in liposomes composed of *E. coli* phospholipid/egg phosphatidylcholine (3:1), the activity of the GlnPQ transporter was highest when the external medium was hyperosmotic relative to the inside (Fig. 5A). Both salts (NaCl and KCl) and membrane-impermeant nonionic osmolytes (sucrose) stimulated the uptake to the same extent when added in equiosmolar concentrations (the membrane-permeant nonionic osmolyte glycerol had no effect), which is reminiscent of the activation of the osmoregulatory glycine betaine transporter OpuA from *L. lactis*. In the case of OpuA, the osmotic activation originates from the increase in luminal electrolyte concentration upon osmotic upshift, converting the osmotic signal into an ionic signal, which is sensed by the transporter through altered lipid-protein interactions (4, 12). Comparable increases in electrolyte concentration upon osmotic upshift take place in the proteoliposomes with GlnPQ. To determine whether possibly a similar osmoregulatory mechanism is operative in GlnPQ, we reconstituted the transporter complex in synthetic lipid mixtures (DOPC/DOPE/DOPG) with varying fractions of the anionic lipid DOPG and determined the osmotic activation profiles. Fig. 5B shows that the fraction of DOPG influenced the maximal activity of GlnPQ

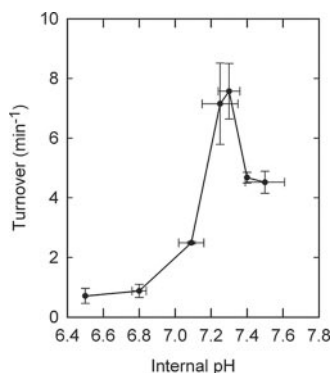


FIG. 6. **Internal pH dependence of glutamine uptake.** The internal pH was varied by diluting proteoliposomes in 50 mM potassium phosphate, pH 6.5, 100 mM potassium acetate into media consisting of 50 mM potassium phosphate plus varying ratios of potassium acetate and K-MES (pH 6.5); the final external potassium acetate concentrations were 100, 30, 10, 5, 1.5, and 1 mM. The internal pH was determined from the fluorescence of pyranine. Uptake of ³H-glutamine (final concentration 20 μ M) was assayed after diluting 5 μ l of proteoliposomes (40 mg/ml lipids) from ice into 195 μ l of buffer with varying ratios of potassium acetate/K-MES at 30 °C.

(the optimum being ~34% DOPG), but the osmotic activation profiles were very similar in all cases. Without DOPG and in the mixture with 50% DOPE/50% DOPG, the uptake was very low, which is at least partly because of a lower reconstitution efficiency in these lipid mixtures, as deduced from SDS-PAGE (not shown). In all other mixtures, there was no visible variation in the reconstitution efficiency, and the activity differences most likely reflect a dependence of GlnPQ on the luminal electrolyte concentration. From these experiments, we conclude that the anionic lipids are essential for GlnPQ activity, but the lipids do not play a direct role in the ionic activation as proposed for OpuA.

pH Dependence of Glutamine and Glutamate Uptake—The uptake of glutamine and glutamate has been studied in detail for whole cells of *L. lactis*, previously *Streptococcus lactis* ML-3 and *Streptococcus cremoris* Wg2 (5, 6). Transport was found to be dependent on the internal pH, with little or no activity below pH 6 and maximal activity at pH 7.5–8. Moreover, glutamate was found to be transported as protonated species, thus requiring an acidic external pH. The requirement for an alkaline internal and acidic external pH for uptake of glutamic acid poses severe limitations on the possibility of assaying for transport. Indeed, in proteoliposomes, significant uptake was only observed when pH_{out} was 5.0 and the internal pH was >7.

To determine the internal pH dependence of GlnPQ, proteoliposomes resuspended in a buffer containing 100 mM potassium acetate were diluted into equiosmolar media with different amounts of potassium acetate to vary the acetate diffusion gradients. Because acetic acid (but not the acetate anion) is highly membrane-permeable, the outward diffusion of acetic acid increased the internal pH, according to $pH_{in} = pH_{out} + \log ([potassium\ acetate]_{in}/[potassium\ acetate]_{out})$. The rate of uptake of [³H]glutamine at an external pH of 6.5 was determined under conditions where initially (time zero, at proteoliposome dilution) the internal pH was 0–1.0 pH unit higher than the outside pH, as determined from pyranine fluorescence measurements. The rate of uptake increased with increasing internal pH and displayed an optimum of ~7.3 (Fig. 6). Control experiments showed that the ATP concentration inside the proteoliposomes was constant and ~9 mM during the course of the assay. The relatively large error in the experiments, as shown in Fig. 6, reflect the initial drop in internal pH upon dilution of potassium acetate-loaded proteoliposomes, which affected the transport rate (because of the pH dependence of

the system) and the determination of the internal pH. However, three independent sets of experiments, performed at external pH values between pH 6 and 7, clearly revealed the internal pH dependence of GlnPQ.

DISCUSSION

The glutamine-glutamic acid transporter from *L. lactis* IL1403 is a member of the PAO family of the ABC superfamily with a unique domain structure. The GlnP protein has two different SBDs fused in tandem at the amino-terminal side of the translocator domain. The first substrate-binding domain is preceded by a signal sequence, which thus far has only been shown for one other prokaryotic membrane protein, the ammonia transporter AmtB from *E. coli* (13). Whereas the function of the signal sequence in AmtB is unknown, in the case of GlnPQ, it serves a role in directing translocation across the membrane of the two SBDs.

The two SBDs of GlnPQ are 50% identical, and SBD1 and SBD2 are 32 and 34% identical to the periplasmic substrate-binding protein GlnH from *E. coli*. The structures of GlnH in the open and liganded form have been determined (8, 9), and most of the residues in the substrate-binding pocket of GlnH are conserved in SBD1 and SBD2 of GlnPQ. Our experiments indicate that, surprisingly, deletion of the first SBD has little or no effect on the kinetics of glutamine uptake, whereas the deletion of the second SBD is detrimental for transport. Because SBD1 has been preserved through evolution, it may have a regulatory role, although not directly participating in the transport cycle. However, functional analysis of wild-type GlnPQ and GlnPQ Δ SBD1 did not reveal noticeable differences in the specificity of transport; only the turnover number was somewhat reduced. In contrast to the glycine betaine transporter OpuA with two identical SBDs in the functional complex (14), we did not observe any cooperativity in the substrate dependence of uptake mediated by GlnPQ or GlnPQ Δ SBD1. The fact that wild-type GlnPQ and both deletion mutants were produced in similar amounts, behaved protein chemically very similarly (each complex was expressed in the membrane and had no tendency to aggregate after solubilization and/or purification), and were highly similar in sequence makes us believe that not only SBD2 in GlnPQ Δ SBD1 but also SBD1 in GlnPQ Δ SBD2 are correctly folded. In respect to the failure to detect any transport activity of the GlnPQ Δ SBD2 mutant, we note that it may be more tedious to engineer an internal (Δ SBD2) than amino-terminal deletion (Δ SBD1) and allow the remaining domain to functionally interact with the translocator. However, as detailed under “Results” and Fig. 1B, both deletion mutants were carefully designed, and it is unlikely that the linker region in GlnPQ Δ SBD2 is too short to allow SBD1 to donate the substrate to the translocator. Future studies are aimed at the further elucidation of the roles of the different SBDs in the transport process and/or physiology of the cell by structure-guided site-directed mutagenesis of SBD1 and SBD2.

Recently, it has been observed that the *glnPQ* genes of group B streptococci are playing a role in virulence (15). This GlnPQ transporter is homologous to the *L. lactis* system studied here. For GlnPQ from group B streptococci, it has been proposed that the transporter plays a role in the regulation of expression of fibronectin adhesins and virulence of this pathogen. Whether GlnPQ from *L. lactis* has analogous regulatory roles remains to be determined.

Disruption of the *glnPQ* gene resulted in the loss of glutamine and glutamic acid uptake, and the cells were no longer able to grow on chemically defined medium with glutamine or glutamate as the sole source of glutamine or glutamate. The disruption mutant grew normally when glutamine was added

to the medium in the form of a dipeptide (Ala-Gln). Here, one of the dipeptide transport systems of *L. lactis* is used to compensate for the loss of Gln/Glu transport function. The GlnPQ transporter is therefore the only transporter for glutamine (and glutamic acid) uptake in *L. lactis*.

The GlnPQ complex is responsible for glutamine and glutamate transport, but the latter is only transported in the protonated form, which is glutamic acid. Previously (6), the surprising observation was made that arginine inhibited the uptake of glutamine in whole cells with an apparent K_i in the submillimolar range. Using purified and membrane-reconstituted GlnPQ, we now confirm that arginine competitively inhibits Gln uptake with a K_i of ~ 2 mM. Except for glutamic acid, arginine, and γ -glutamylhydrazide, there was no clear inhibition of glutamine uptake by any of the other amino acids tested. The substrate specificity of GlnPQ and GlnPQASBD1 were very similar and seems to be determined by SBD2. The amino acid-binding site of SDB2 seems to tolerate fairly large neutral and basic side chains. Asparagine and aspartic acid, on the other hand, residues that are only one methylene shorter than glutamine and glutamic acid, did not inhibit the transport. Thus, the positioning of the amide and carboxylic acid moiety is crucial for high affinity transport. In contrast to lysine, arginine is competing with glutamine, although with a low affinity. It is possible that the guanidinyll moiety in arginine makes critical contacts with the protein, which are not possible with lysine. In respect to the region in the binding pocket of SBD2 that accommodates the side-chain amine moiety of glutamine or the hydroxyl of the glutamic acid, larger functionalities are also tolerated, which are the hydrazide of γ -glutamylhydrazide and even (part of) the bulky guanidinyll of arginine. However, for binding (and transport) of substrate, it is prerequisite that the overall charge is neutral (high affinity transport) or positive (low affinity competition by arginine).

The uptake of glutamine in proteoliposomes was highly stimulated when the concentrations of ionic osmolytes on the luminal side of the membrane were increased, *i.e.* by osmotic upshift. These observations mimic the activation of the osmoregulatory OpuA from *L. lactis*, ProP from *E. coli*, and BetP from *Corynebacterium glutamicum* (4, 12, 16, 17) and contrast with that of other, non-osmoregulatory (ABC) transporters, such as Opp.² Because osmosensing by the OpuA system is mediated by anionic lipid-protein interactions (4), it was important to investigate the osmotic activation of the GlnPQ complex and to determine the (anionic) lipid dependence of the system. Although there was an optimal DOPG concentration for maximal activity of GlnPQ, the activation profiles were very similar at every DOPG concentration, which contrasts the situation for OpuA, where the activation profiles shift to higher osmolalities with increasing fractions of anionic lipids in the membrane. Thus, the osmotic activation of GlnPQ seems to be a direct consequence of the concentrated (ionic) osmolytes at the luminal ("cytoplasmic") side of the membrane on the protein, rather than a modulation of (anionic) lipid-protein interactions. Even though this mode of activation is different from that described for OpuA (3, 4), it would allow the cell to accumulate faster (and more) glutamine and glutamate when it is confronted with hyperosmotic stress, wherein the cytoplasmic volume decreases and the ion concentration (ionic strength) increases as a consequence of water efflux.

Recently, we proposed a common physicochemical mechanism of osmosensing (12), which for OpuA has been modeled as an on/off electrostatic lever that assumes "electrostatically locked" or "thermally relaxed" conformations. The on/off activation was found to correlate with the theoretical prediction of transitions in ionic clouds according to the Maxwellian version of the Poisson-Boltzmann equation. A similar electrostatic mechanism may be operative in GlnPQ, except that screened electrostatic forces playing a role in the different conformational states of the transporter are not mediated by lipid-protein interactions but only involve protein domains and low molecular weight electrolytes. Because the pH inside the proteoliposomes influences the ionization state of GlnPQ and low molecular weight electrolytes (phosphate, creatine-phosphate, ATP, and ADP in our experimental setup), it is well possible that the internal pH and salt dependence of GlnPQ activity have a common electrostatic basis.

How relevant is the observed regulation of GlnPQ for *L. lactis*? Molenaar *et al.* (18) measured an increase in the pool of glutamate after growing *L. lactis* ML3 under hyperosmotic conditions, implying that glutamate could serve as an osmoprotectant in this organism. Similar to OpuA, a decrease in cell volume and the accompanying increase in ion concentration upon osmotic upshift could activate GlnPQ *in vivo*, and the further accumulation of glutamate (up to ~ 50 mM) would offset the deleterious effects of the stress. However, we emphasize that the role of OpuA in osmoprotection is far greater than that of GlnPQ (glycine betaine is accumulated to >0.5 M, whereas glutamate plus glutamine reach levels of at most (50 mM), which may also be reflected in the more advanced electrostatic switching mechanism of OpuA. The activity of OpuA is modulated over 2–3 orders of magnitude, whereas regulation of GlnPQ is <10 -fold.

Acknowledgments—We thank Esther Biemans-Oldehinkel, Sytse Henstra, and Mark Doeve for stimulating discussions.

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² M. K. Doeve, unpublished observations.